

Glucosamine Activates the Plasminogen Activator Inhibitor 1 Gene Promoter Through Sp1 DNA Binding Sites in Glomerular Mesangial Cells

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Increased flux through the hexosamine biosynthetic pathway is associated with altered gene expression. To investigate the underlying mechanisms, we treated glomerular mesangial cells with glucosamine and studied the regulation of the plasminogen activator inhibitor (PAI)-1 gene. Incubating mesangial cells with 2 mmol/l glucosamine for 4 days resulted in a 3.1 ± 0.4 -fold increase in PAI-1 mRNA levels ($P < 0.01$) and a 33 ± 9 -fold increase in the activity of a transiently transfected PAI-1 promoter-luciferase reporter gene ($P < 0.01$). Cotransfection of an expression vector for a dominant-negative type II TGF- β receptor with the PAI-1 promoter-reporter gene did not interfere with this effect of glucosamine. However, mutation of 2 putative Sp1 sites in the PAI-1 promoter, at -76 to -71 and -44 to -39, markedly reduced induction of PAI-1 luciferase activity by glucosamine, from 8.9 ± 1.9 -fold to 1.7 ± 0.5 -fold ($P < 0.01$). An electrophoretic mobility shift assay demonstrated that glucosamine increased Sp1 DNA binding by $31 \pm 11\%$ ($P < 0.05$), implying that the effects of glucosamine were explained, in part, by changes in Sp1 DNA binding. High glucose (20 mmol/l) also activated the transiently transfected PAI-1 promoter (2.5 ± 0.4 -fold). This effect was diminished by mutation of both the PAI-1 promoter Sp1 sites (1.2 ± 0.3 -fold, $P < 0.05$). In addition, 6-diazo-5-oxo-L-norleucine, a glutamine:fructose-6-phosphate-amidotransferase inhibitor, blocked the induction by high glucose (4.7 ± 0.8 - to 0.9 ± 0.1 -fold, $P < 0.01$). These results indicate that stimulation of the PAI-1 promoter by both high glucose and glucosamine involves Sp1 and that the hexosamine pathway may be involved in the regulation of gene expression by high glucose in glomerular mesangial cells. *Diabetes* 49:863-871, 2000

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Received for publication 10 September 1999 and accepted in revised form 3 February 2000.

DMEM, Dulbecco's modified Eagle's medium; DON, 6-diazo-5-oxo-L-norleucine; DTT, dithiothreitol; ECM, extracellular matrix; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; GFA, glutamine:fructose-6-phosphate-amidotransferase; O-GlcNAc, O-linked N-acetylglucosamine; PAI, plasminogen activator inhibitor; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; SSC, sodium chloride-sodium citrate; TGF, transforming growth factor; UDP-GlcNAc, UDP-N-acetylglucosamine; UDP-HexNAc, UDP-N-acetylhexosamine.

Prolonged hyperglycemia is one of the major determinants of long-term complications of diabetes (1). Among these complications, diabetic glomerulosclerosis is characterized by the accumulation of extracellular matrix (ECM) proteins in the glomerular mesangium, impairing capillary flow and ultimately resulting in glomerular destruction and progressive renal failure (2,3). Increases in the expression of genes encoding ECM proteins (such as fibronectin, type IV collagen, and laminin) and regulators of ECM deposition (such as transforming growth factor [TGF]- β) induced by high concentrations of glucose are thought to underlie diabetic glomerulosclerosis (4-6).

Cultured glomerular mesangial cells have been used as a model to study the mechanisms of these high glucose-mediated changes in gene expression. In these cells, high glucose causes increased expression of the type IV collagen and fibronectin genes, mimicking the in vivo alterations (7-10). Several metabolic effects of high glucose have been postulated to contribute to its impact on gene expression, including de novo diacylglycerol synthesis and activation of protein kinase C (PKC) (7,11,12), increased activity of the polyol pathway (11), oxidative stress (13), and the formation of advanced glycation products (11). Glomerular hyperfiltration (14) and augmented release of TGF- β (8,15) are secondary outcomes that add to the effects of these metabolic abnormalities. Recently, one other metabolic effect of high glucose, increased flux through the hexosamine biosynthesis pathway, has been postulated to be a mediator of glucose-induced changes in gene expression.

About 1-3% of glucose is normally diverted through the hexosamine biosynthesis pathway, whereby fructose-6-phosphate is converted by the rate-limiting enzyme, glutamine:fructose-6-phosphate-amidotransferase (GFA), to glucosamine-6-phosphate (16). The end product of this pathway, UDP-N-acetylglucosamine (UDP-GlcNAc), is a substrate for protein glycosylation (16). The potential importance of the hexosamine pathway as a high glucose effector was first suggested by studies of insulin resistance. Thus it was found that glutamine was necessary for the induction of insulin resistance by high glucose (17). Furthermore, glucosamine mimicked glucose in causing insulin resistance. Although high glucose-induced insulin resistance was blocked by inhibition of GFA, this did not occur in the case of glucosamine, which promotes flux through the hexosamine pathway distal to this enzyme (17). Similarly, infusion of glucosamine into rats or overexpression of GFA in cultured cells or in trans-

genic mice was found to result in insulin resistance (18–21). Of interest, infusion of glucose, free fatty acids, uridine, or glucosamine into rats, each of which elevated levels of UDP-GlcNAc, increased leptin gene expression in muscle (22). These results suggest that the hexosamine pathway functions as a cellular sensor of nutrient availability by modulating gene expression in peripheral insulin target tissues (22).

Importantly, the hexosamine biosynthesis pathway also appears to modulate gene expression in non-insulin-sensitive tissues. For example, both high glucose and glucosamine stimulated TGF- α gene expression, and this effect of high glucose was blocked by chemical inhibition of GFA or by expression of antisense RNA directed against GFA (23,24). More recently, Kolm-Litty et al. (25) demonstrated that glucosamine enhanced the synthesis of TGF- β , type IV collagen, fibronectin, and proteoglycans in cultured porcine mesangial cells. We have also found in preliminary studies (26) that glucosamine increased TGF- β 1 and type II TGF- β receptor mRNA levels in rat mesangial cells. Although GFA is not detectable in normal adult kidney by immunohistochemical methods, it is present in substantial amounts in mesangial and epithelial cells of glomeruli from patients with diabetic nephropathy and mesangial cells in tissue culture (27).

These observations raise the possibility that flux through the hexosamine biosynthesis pathway is elevated in mesangial cells in diabetes and that this enhanced flux contributes significantly to the pathogenesis of diabetic nephropathy by altering gene expression. To determine the mechanisms whereby the hexosamine pathway affects gene expression, glomerular mesangial cells were transiently transfected with a plasminogen activator inhibitor (PAI)-1 promoter/luciferase reporter gene and exposed to glucosamine. The expression of PAI-1 is induced by angiotensin II, TGF- β , and PKC, mediators of diabetic nephropathy (28–30). Moreover, PAI-1 gene expression has been shown to be regulated at the level of transcription, and the 5'-flanking elements in the PAI-1 gene promoter have been well characterized (28,30–32). Exogenous glucosamine was used to increase flux through the hexosamine pathway. This approach revealed that one of the targets of both high glucose and glucosamine in mesangial cells is the transcription factor Sp1.

RESEARCH DESIGN AND METHODS

Cell culture. Rat glomerular mesangial cells were isolated from Sprague-Dawley rats, characterized as previously described (33,34), and grown in Dulbecco's modified Eagle's medium (DMEM) (D-glucose concentration 5.6 mmol/l) supplemented with 17% fetal bovine serum (Life Technologies, Grand Island, NY) and 1% penicillin/streptomycin. Experiments were conducted with cells between passages 19 and 22.

Plasmids. Construction of the luciferase reporter gene containing the -740 to 44 human PAI-1 promoter in the vector, pA₃LUC, has been described (35). The expression vector for the dominant-negative type II TGF- β receptor contained the cDNA for the extracellular and transmembrane domains (amino acids 1–221) of the rat type II TGF- β receptor. This cDNA was obtained by polymerase chain reaction (PCR) from a rat brain cDNA library using the high-fidelity DNA polymerase, pfu (Stratagene, La Jolla, CA), and inserted into the expression vector, pcDNA3.1/myc-His C (Invitrogen, Carlsbad, CA). This and other sequences synthesized by PCR were confirmed by dideoxy DNA sequencing. Mutations in both Sp1 sites in the PAI-1 promoter at -75 to -71, changing 5'-GGTGG-3' to 5'-AATTC-3', and -44 to -39, changing 5'-CTGCC-3' to 5'-TAGAT-3', as described by Chen et al. (36), were generated by PCR-based site-directed mutagenesis and verified by restriction enzyme digestion and sequencing.

Transfection. DNA was introduced into subconfluent mesangial cells grown in 24-well plates using the lipid-based transfection reagent Fugene-6 (Roche Molecular Biochemicals, Indianapolis, IN), according to the manufacturer's

instructions. Each well received a total of 0.5 μ g DNA and 0.83 μ l Fugene-6. Glucosamine (2 mmol/l) (Sigma, St. Louis, MO), freshly prepared and adjusted to pH 7.4, was added to standard DMEM containing 5.6 mmol/l glucose at the time of cell plating as indicated. No increase in trypan blue staining was observed after a 4-day exposure to 2 mmol/l glucosamine (not shown). For the high glucose experiments, cells were incubated in DMEM containing either 20 or 1 mmol/l D-glucose. Two days posttransfection, the medium was changed to serum-free medium (minimal essential medium with 0.1% albumin, 20 mmol/l HEPES, pH 7.4, 5.6 mmol/l D-glucose), and fresh glucosamine was added to the cells. After a further 24 h, the mesangial cells were washed 3 times with phosphate-buffered saline (PBS) and lysed for 30 min on ice in 100 mmol/l KH₂PO₄, pH 7.9, and 0.5% Triton X-100. Luciferase was measured in 50 μ l extract with 300 μ l buffer containing 100 mmol/l Tris, pH 7.8, 10 mmol/l MgSO₄, 2 mmol/l EDTA, 5.5 mmol/l ATP, 1 mmol/l dithiothreitol (DTT), and 50 μ mol/l luciferin in a Berthold Lumat LB 9501 luminometer (E.G. and G. Berthold, Vienna, Austria). Results were normalized to protein content, determined by modified Lowry assay using a Bio-Rad protein DC kit (Bio-Rad, Hercules, CA). All transfections were repeated at least 3 times, and the results were calculated as fold-stimulation relative to basal values \pm SE. In preliminary experiments, it was found that correcting for transfection efficiency by cotransfecting β -galactosidase expression vectors driven by viral promoters gave misleading results. The activity of these promoters was stimulated by glucosamine, perhaps owing to the presence of Sp1 sites (or sites indirectly responsive to Sp1) in the viral promoters. A similar problem was encountered in studies of the von Hippel-Lindau gene product, which interacts with Sp1 (37).

Nuclear extract preparation and electrophoretic mobility shift assay. Nuclear extracts were prepared from mesangial cells plated in 10-cm dishes, essentially as described by Dignam et al. (38). Cells were washed 3 times with PBS, scraped into 10 mmol/l HEPES, pH 7.9, 1.5 mmol/l MgCl₂, 10 mmol/l KCl, 0.5 mmol/l DTT, 0.2 mmol/l phenylmethylsulfonyl fluoride (PMSF), 20 μ g/ml aprotinin, and 100 nmol/l microcystin LR; lysed on ice for 10 minutes; and homogenized with a Dounce homogenizer (Fisher Scientific, Pittsburgh, PA). Nuclei were pelleted; resuspended in 20 mmol/l HEPES, pH 7.9, 25% glycerol, 420 mmol/l NaCl, 1.5 mmol/l MgCl₂, 0.2 mmol/l EDTA, 0.5 mmol/l DTT, 2 mmol/l PMSF, 20 μ g/ml aprotinin, and 100 nmol/l microcystin LR; kept on ice for 20 min; and centrifuged again for 5 min at 10,000g to eliminate debris. The supernatants were used as crude nuclear extracts. Protein concentration was measured with a protein assay kit (Bio-Rad). Double-stranded oligonucleotides were prepared by annealing single-stranded oligonucleotides containing the putative Sp1 A site (-85 to -63) from the human PAI-1 promoter (36) (sense: 5'-CAGT GAGTGGGTGGGGCTGGAAC-3', antisense: 5'-GTTCCAGCCCCACCACTCA CTG-3') and labeled with [γ -³²P]ATP using polynucleotide kinase. Electrophoretic mobility shift assays (EMSA) were performed by incubating 5–10 μ g crude nuclear extract with 20,000 cpm [³²P]-end-labeled double-stranded oligonucleotide (0.1 ng) in binding buffer (10 mmol/l HEPES, pH 7.9, 10 mmol/l KCl, 1 mmol/l EDTA, 5 mmol/l MgCl₂, 5% glycerol, 1 mmol/l ZnCl₂, 0.5 mmol/l DTT, 2 mmol/l PMSF, 20 μ g/ml aprotinin, and 100 nmol/l microcystin LR) for 20 min at room temperature. Unlabeled competitor double-stranded oligonucleotides were added in 100-fold molar excess for 10 min at room temperature before the above incubation, as indicated. For supershift experiments, 4 μ g antibodies against Sp1 (sc-59 X) or Sp3 (sc-644 X) (both from Santa Cruz Biotechnology, Santa Cruz, CA) or normal rat immunoglobulin was added to the nuclear extracts for 20 min at room temperature before the binding reaction. DNA-protein complexes were resolved on a 5% acrylamide gel by electrophoresis in 0.5 \times TBE (1 \times TBE is 90 mmol/l Tris, 90 mmol/l boric acid, and 2 mmol/l EDTA, pH 8) at 100 V. The gel was subsequently dried and visualized with a Storm 840 Phosphorimager (Molecular Dynamics, Sunnyvale, CA) and ImageQuant 5.0 software (Molecular Dynamics).

ATP assay. Mesangial cells were grown for 1–4 days in the presence or absence of 2 mmol/l glucosamine. The cells were washed 3 times with PBS and scraped directly into somatic cell ATP-releasing reagent (Sigma). The ATP content of these extracts was determined with an ATP bioluminescence assay kit (Sigma) using a luciferin/luciferase reaction (39) in a Berthold Lumat LB 9501 luminometer, according to the manufacturer's instructions.

Northern blot analysis. Total cellular RNA was prepared from subconfluent mesangial cells grown in 10-cm dishes using the acid guanidinium-phenol procedure of Chomczynski and Sacchi (40). Denatured RNA (5 μ g/lane) was separated on a 1% agarose gel in 1 \times TBE containing 20 mmol/l guanidine isothiocyanate, transferred to GeneScreenPlus membranes (Dupont/New England Nuclear, Boston, MA), and cross-linked to the membrane with 50 mmol/l NaOH. The rat PAI-1 probe (41), a gift of Dr. Allison Eddy (Washington University, Seattle, WA), was labeled with [α -³²P]dCTP using a T7 polymerase kit (Amersham/Pharmacia, Piscataway, NJ). The blots were prehybridized for 1 h in 200 mmol/l NaH₂PO₄, 300 mmol/l Na₂HPO₄, pH 7, 7% SDS, 1 mmol/l EDTA, 1% bovine serum albumin, 1 mmol/l Na₄P₂O₇, and 125 μ g/ml salmon sperm DNA

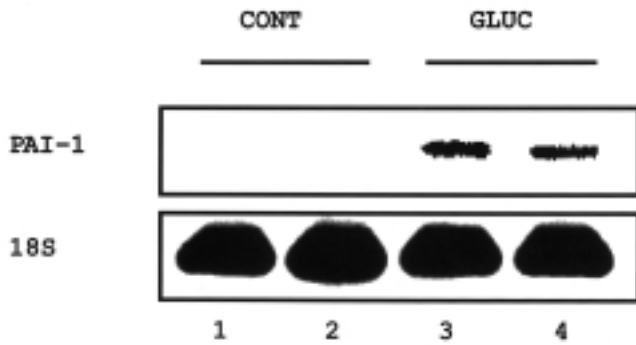


FIG. 1. Glucosamine increases PAI-1 mRNA levels. A: Mesangial cells were exposed to 2 mmol/l glucosamine (GLUC, lanes 3 and 4) or left untreated (CONT, lanes 1 and 2) for 4 days. Total RNA was extracted and 5 μ g was analyzed by Northern blotting using a rat PAI-1 cDNA probe. The blot was reprobed with an 18S RNA probe to control for loading. Results shown are representative of 5 experiments.

and hybridized overnight at 60°C in the same buffer with 1×10^6 cpm/ml radio-labeled probe in a hybridization oven. The blots were then washed with $2 \times$ sodium chloride-sodium citrate (SSC), 0.1% SDS at room temperature for 5 and 20 min; $2 \times$ SSC, 0.1% SDS at 60°C for 20 min; $0.2 \times$ SSC, 0.1% SDS at 60°C for 45 min; and $2 \times$ SSC at room temperature for 5 min ($1 \times$ SSC is 150 mmol/l sodium chloride, 15 mmol/l sodium citrate, pH 7) and analyzed with a phosphorimager (as above). Blots were reprobed with a mouse 18S ribosomal RNA probe (a gift from Dr. D. Drucker, University of Toronto, Ontario, Canada) to normalize for RNA loading.

Data analysis. Statistical significance was determined using Student's *t* test with the program Statistica (Statsoft, Tulsa, OK). Differences were considered significant at $P < 0.05$. Results are expressed as mean \pm SE

RESULTS

Glucosamine increases PAI-1 mRNA levels and PAI-1 gene promoter activity. We first assessed the effects of glucosamine on PAI-1 mRNA levels by Northern blot analysis. Incubation of mesangial cells with 2 mmol/l glucosamine, a relatively low concentration to avoid potentially toxic effects such as nucleotide depletion (see below), for 4 days resulted in a significant 3.1 ± 0.4 -fold ($P < 0.01$) increase in PAI-1 mRNA levels (Fig. 1). To determine whether this increase in PAI-1 mRNA was associated with an enhanced transcriptional activity, mesangial cells were transiently transfected with a plasmid containing the PAI-1 gene promoter fused to a luciferase reporter gene. Exposure to 2 mmol/l glucosamine caused a time-dependent increase in luciferase activity, with a marked (33 ± 9 -fold) increase after 4 days and a significant 4.5 ± 0.9 -fold increase after 3 days but little change after 1 or 2 days (Fig. 2). The 4-day time point was chosen for further studies. This increase in luciferase activity was dose dependent and peaked at 2 mmol/l glucosamine (not shown). Higher concentrations resulted in a decrease in cell growth and transfection efficiency. The effect of glucosamine on the transiently transfected PAI-1 reporter gene exceeded that on endogenous PAI-1 mRNA levels. This difference could be explained by baseline PAI-1 mRNA levels being higher than luciferase mRNA levels at the start of the experiment and therefore less subject to change. Additionally, regulatory elements could be present in the endogenous gene, but not in the fragment of the PAI-1 promoter that we studied.

Glucosamine activates the PAI-1 promoter independently of TGF- β . Because glucosamine increases TGF- β 1 synthesis (25,26) and TGF- β has been shown to increase PAI-1 promoter activity (28,35), it was possible that glu-

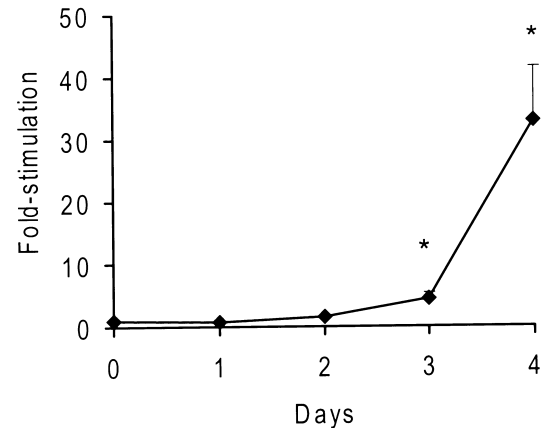


FIG. 2. Stimulation of PAI-1 promoter activity by glucosamine is time dependent. Mesangial cells were transiently transfected with a luciferase reporter gene containing the PAI-1 promoter (-740 to 44) and treated for 1-4 days with 2 mmol/l glucosamine or left untreated. Luciferase was assayed after 24 h in serum-free medium. The results are expressed as fold-stimulation by glucosamine compared with unstimulated cells. Values are means \pm SE ($n = 5$). * $P < 0.05$ vs. untreated.

cosamine was acting indirectly on the PAI-1 promoter through TGF- β . To evaluate this possibility, an expression vector encoding a dominant-negative type II TGF- β receptor, which has its cytoplasmic domain truncated, was cotransfected with the PAI-1 reporter gene. This type of dominant-negative construct has previously been shown to block TGF- β -initiated signal transduction by preventing phosphorylation of type I TGF- β receptors by type II TGF- β receptors (42). Expression of this dominant-negative receptor reduced activation of the PAI-1 promoter by TGF- β from 4.4 ± 1.1 - to 1.2 ± 0.2 -fold ($P < 0.05$) (Fig. 3) but did not impair stimulation of PAI-1 promoter activity by glucosamine. There was a 29 ± 7 -fold activation by glucosamine when the empty vector was cotransfected compared with 36 ± 12 -fold activation when the dominant-negative TGF- β receptor was coexpressed (Fig. 3). Therefore, glucosamine exerts effects on the PAI-1 promoter in mesangial cells that appear to be independent of TGF- β .

Glucosamine acts on the PAI-1 promoter via the transcription factor Sp1. High glucose has been previously demonstrated to upregulate PAI-1 promoter activity in vascular smooth muscle cells through 2 Sp1 elements encompassing the promoter segments -76 to -71 and -44 to -39 (36). The ability of glucosamine to increase the cellular levels of UDP-GlcNAc is well established (19,25,43,44), and the transcription factor Sp1 is subject to glucosamine-induced *O*-linked glycosylation (45,46). In addition, Sp1 was suggested, but not documented, to bind to the portion of the TGF- α promoter influenced by glucosamine in vascular smooth muscle cells (23). Therefore, we examined the role of Sp1 in activation of the PAI-1 promoter by glucosamine. Point mutations, which had been demonstrated by Chen et al. (36) to abolish Sp1 DNA binding, were introduced into both of the above PAI-1 promoter Sp1 sites. Upon supplementation with 2 mmol/l glucosamine, there was 8.9 ± 1.9 -fold activation of the transiently transfected wild-type PAI-1 promoter compared with 1.7 ± 0.5 -fold activation for the Sp1 mutant PAI-1 promoter ($P < 0.01$) (Fig. 4). However, stimulation of PAI-1 promoter activity by TGF- β was minimally affected by the Sp1 mutations (3.9 ± 0.9 - to 3.2 ± 0.5 -fold, NS) (Fig. 4), demonstrating that

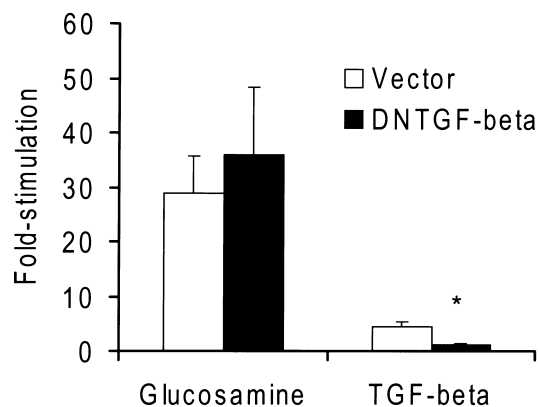


FIG. 3. Stimulation of PAI-1 promoter activity by glucosamine is independent of TGF- β . Mesangial cells were transiently transfected with a -740 to 44 PAI-1 promoter-luciferase reporter gene and either an expression vector for type II dominant-negative TGF- β receptor (DNTGF- β) or the corresponding empty vector (vector). The cells were treated with 2 mmol/l glucosamine for 4 days, 20 ng/ml TGF- β (TGF- β) for 1 day, or vehicle (control). Luciferase activity was assayed after 24 h in serum-free medium. The results are expressed as fold-stimulation by glucosamine or TGF- β compared with control. Results are means \pm SE ($n = 3$). * $P < 0.05$, TGF- β stimulation of cells with DNTGF- β receptor vs. vector alone.

the mutant promoter is able to respond to extracellular stimuli. It may be noted that the fold induction of luciferase by glucosamine was less dramatic in these experiments (Fig. 4) compared with that observed in Figs. 2 and 3. This decrease may reflect slight differences in rates of cell growth or in basal luciferase expression over time. The results, however, were very consistent in each set of experiments.

GC-rich promoter elements are able to interact with a number of zinc finger transcription factors, including members of the Sp1, Kruppel-like factor, and TGF- β -induced early gene families (47–50). To determine whether glucosamine affects the DNA binding activity of Sp1, we performed EMSAs using an oligonucleotide spanning the -85 to -63 region of the PAI-1 promoter, which contains one of the Sp1 sites mutated in the above experiments. Nuclear extracts were prepared from mesangial cells challenged with 2 mmol/l glucosamine or left untreated for 4 days. As illustrated in Fig. 5A, 2 specific DNA protein complexes were formed with this oligonucleotide and mesangial cell nuclear extracts. These complexes, labeled 1a, 1b, and 2, were abolished by competition with a 100-fold molar excess of unlabeled oligonucleotide (Fig. 5A, lane 5). In glucosamine-treated mesangial cells, the DNA binding activity of these extracts was enhanced by an average of $31 \pm 11\%$ ($P < 0.05$) in 5 separate experiments (Fig. 5A, lanes 3 and 4 vs. lanes 1 and 2). EMSAs performed with anti-Sp1 and anti-Sp3 antibodies demonstrated that most of the observed DNA protein complex was supershifted by Sp1 antibodies (Fig. 5B). Sp3 antibodies reduced the formation of DNA protein complexes, but a supershifted band was not visualized (Fig. 5B). These data indicate that glucosamine increased Sp1 binding activity in mesangial cells and that the -85 to -63 PAI-1 promoter oligonucleotide bound predominantly to Sp1.

Role of nucleotide depletion. Incubating cells with glucosamine has been reported to cause variable degrees of cellular ATP and UTP depletion (51–53). Indeed, a reduction of

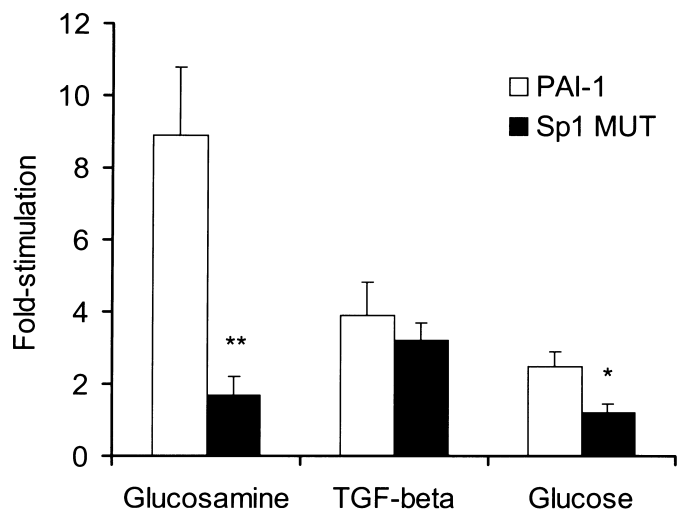


FIG. 4. Sp1 sites are required for activation of the PAI-1 promoter by glucosamine and by high glucose. Mesangial cells were transiently transfected with a luciferase reporter gene containing either the wild-type PAI-1 promoter (-740 to 44) or a PAI-1 promoter with Sp1 sites (-76 to -71 and -44 to -38) mutated (Sp1 MUT). The cells were left untreated or treated with 2 mmol/l glucosamine for 4 days, 20 ng/ml TGF- β or vehicle for 1 day, or 20 mmol/l glucose or 1 mmol/l glucose (control) for 4 days. Luciferase was assayed after 24 h in serum-free medium. The results are expressed as fold-stimulation by glucosamine, TGF- β , or 20 mmol/l glucose compared with untreated, vehicle-treated, or 1 mmol/l glucose-treated cells, respectively, for each promoter. Results shown are means \pm SE ($n = 3$). * $P < 0.05$; ** $P < 0.01$ stimulation with wild-type PAI-1 promoter vs. Sp1MUT.

up to 50% of total cellular ATP was found in cultured 3T3-L1 adipocytes exposed to glucosamine in the presence of insulin and 5 mmol/l glucose, but in the absence of insulin, only mild (10–15%) ATP depletion was observed (51). Hresko et al. (51) postulated that glucosamine-induced ATP depletion was due to phosphorylation of glucosamine by hexokinase. Glucosamine-induced UTP depletion has been attributed to the consumption of UTP during the formation of UDP-GlcNAc (43,53). Nucleotide depletion modulates intracellular signaling under some circumstances. For example, uncoupling of mitochondrial oxidation by dinitrophenol results in increased glucose uptake in L6 muscle cells due to changes in PKC activity and cytosolic calcium (54). To test whether nucleotide depletion occurred under our experimental conditions, ATP levels were measured with a luciferin/luciferase reaction. These assays showed that ATP levels were not decreased by incubating mesangial cells for 1–4 days with 2 mmol/l glucosamine (Fig. 6). These results exclude direct glucosamine-induced ATP depletion and indicate that the effect of glucosamine on the PAI-1 promoter is not due to cell toxicity. Exogenous uridine is able to reverse glucosamine-induced UTP depletion (53). Figure 7 documents that incubation of mesangial cells with 2 mmol/l glucosamine increased PAI-1 promoter activity by 11.5 ± 2.5 -fold ($P < 0.01$), as expected. Uridine by itself had no effect (1.1-fold, NS), but the combination of uridine and glucosamine strongly increased PAI-1 promoter activity (43 ± 8 -fold, $P < 0.01$ vs. glucosamine) (Fig. 7). These results rule out UTP depletion as the mediator of glucosamine-induced increases in PAI-1 reporter gene activity but suggest that the effects of glucosamine may be limited by UTP availability.

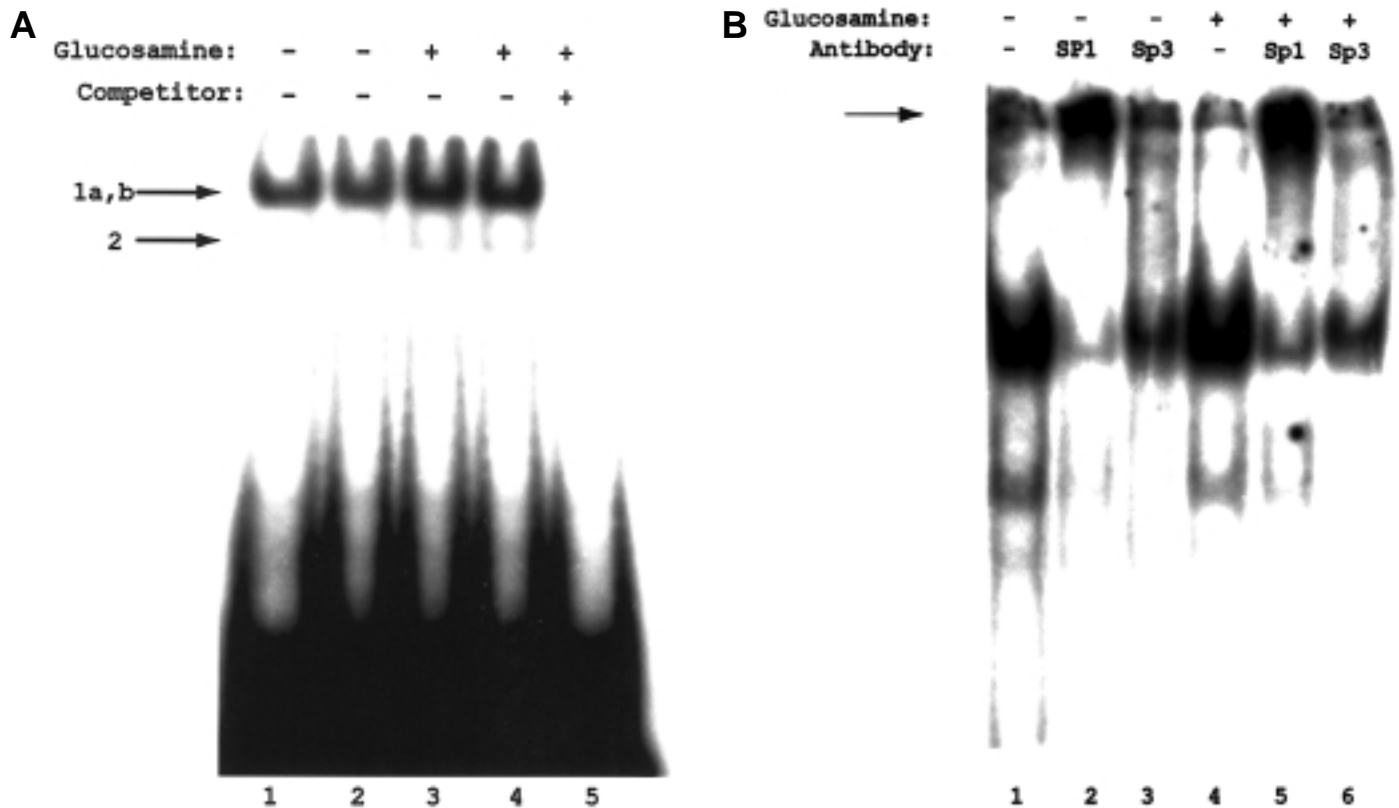


FIG. 5. Sp1 DNA binding activity is increased by glucosamine. **A:** Nuclear extracts were prepared from mesangial cells treated with 2 mmol/l glucosamine for 4 days (*lanes 3 and 4*) or left untreated (*lanes 1 and 2*) and analyzed by EMSA with a [³²P]-labeled oligonucleotide probe (-85 to -63 segment of the PAI-1 promoter). Competition was performed by preincubating nuclear extract from glucosamine-treated cells with a 100-fold excess of unlabeled oligonucleotide (*lane 5*). The arrows indicate complexes noted in other studies of Sp1. **B:** Nuclear extracts were prepared from mesangial cells treated with 2 mmol/l glucosamine for 4 days (*lanes 4-6*) or left untreated (*lanes 1-3*). Extracts were preincubated with specific antibodies for Sp1 (*lanes 2 and 5*), Sp3 (*lanes 3 and 6*), or rabbit immunoglobulin (*lanes 1 and 4*) before analysis by EMSA as in **A**.

High glucose-induced PAI-1 promoter activity is blocked by GFA inhibition and depends on Sp1 sites.

To investigate whether the hexosamine pathway is involved in activation of the PAI-1 promoter by high glucose, we tested the ability of 6-diazo-5-oxo-L-norleucine (DON), a GFA inhibitor (24), to block transactivation of the PAI-1 promoter by high glucose. D-Glucose (1–30 mmol/l) activated the PAI-1 promoter in a dose-dependent fashion, with peak effects at ~5 mmol/l glucose (data not shown). This saturation of glucose effects at a relatively low glucose concentration appears to be a function of this particular mesangial cell culture system. It should be noted that Heilig et al. (55) reported that glucose uptake in mesangial cells, which occurs through the glucose transporter GLUT1, has a K_m of 0.46 mmol/l and becomes saturated at physiological glucose concentrations (8 mmol/l). This implies that the effects of high glucose may be limited by glucose uptake in these cells. We evaluated the effects on the PAI-1 promoter of 20 mmol/l compared with 1 mmol/l glucose. These experiments (Fig. 8) revealed that high glucose stimulated the PAI-1 promoter 4.7 ± 0.8-fold. This effect was completely blocked (0.9 ± 0.1-fold) by 40 μmol/l DON ($P < 0.01$), whereas activation of the PAI-1 promoter by glucosamine was not significantly altered by DON. There was 20.6 ± 3-fold-stimulation observed with glucosamine versus 15.2 ± 3-fold with glucosamine and DON (NS). In contrast, similar to the results obtained with glucosamine, stimula-

tion of the PAI-1 promoter by high glucose (20 mmol/l) was suppressed from 2.5 ± 0.4- to 1.2 ± 0.3-fold ($P < 0.05$) when the PAI-1 promoter Sp1 sites were mutated (Fig. 4).

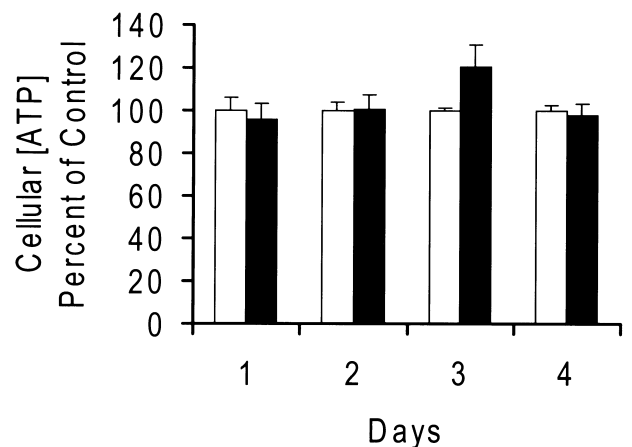


FIG. 6. Effect of glucosamine on mesangial cell ATP levels. Mesangial cells were incubated with 2 mmol/l glucosamine (■) for 1–4 days or left untreated (□) and lysed, and cellular ATP levels were determined with a luciferin/luciferase reaction. The results are expressed relative to cells grown under similar conditions without glucosamine. Results are means ± SE ($n = 3$).

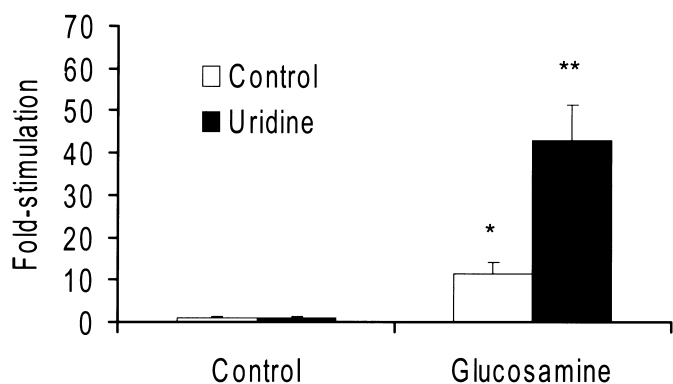


FIG. 7. Uridine enhances glucosamine-induced activation of the PAI-1 promoter. Mesangial cells were transiently transfected with a -740 to 44 PAI-1 promoter-luciferase reporter gene. The cells were treated for 4 days with and without 2 mmol/l glucosamine in the presence (■) and absence (□) of 10 mmol/l uridine. Luciferase was assayed after 24 h in serum-free medium. The results are expressed as fold-stimulation by glucosamine, uridine, or both compared with unstimulated cells. Results are means \pm SE ($n = 3$). * $P < 0.01$, glucosamine vs. untreated; ** $P < 0.01$, glucosamine with uridine vs. glucosamine alone.

DISCUSSION

Enhanced flux through the hexosamine biosynthesis pathway has been implicated as a contributor to the pathogenesis of insulin resistance (17–21,56) and, more recently, to the pathogenesis of diabetic nephropathy (25). These effects on cell function may involve alterations in gene expression. In fact, glucosamine has been previously shown to enhance the expression of the TGF- α , TGF- β , and leptin genes (22–26). In this study, we used the PAI-1 gene promoter to characterize the actions of glucosamine on gene expression and explore the mechanisms in a cell type relevant to diabetic nephropathy, the glomerular mesangial cell (2,3). We first found that treatment of mesangial cells with 2 mmol/l glucosamine increased steady-state PAI-1 mRNA levels. As expected from previous reports (19,25,43,44), we have observed in preliminary experiments that UDP-*N*-acetylhexosamine (UDP-HexNAc) levels (measured by high-performance liquid chromatography) were increased 13-fold in mesangial cells after a 3-day incubation with 2 mmol/l glucosamine (data not shown). In transient transfection experiments, incubating mesangial cells with 2 mmol/l glucosamine strongly activated the PAI-1 promoter after 4 days. The lack of change in ATP levels and the ability of uridine to augment activation of the PAI-1 promoter by glucosamine argue against nucleotide depletion as the explanation for the actions of glucosamine. In addition, we would predict that addition of uridine would also augment the action of glucosamine to increase PAI-1 mRNA levels. Interestingly, disruption of two putative Sp1 binding sites in the PAI-1 promoter blocked induction of PAI-1 promoter activity by glucosamine. EMSAs documented that the -85 to -63 PAI-1 promoter segment bound mainly to Sp1 in mesangial cells and that the DNA binding activity of Sp1 was moderately increased by glucosamine.

This report is the first to formally demonstrate that glucosamine-induced changes in gene expression are mediated to a significant extent through the transcription factor Sp1. Furthermore, stimulation of the PAI-1 promoter by high glucose in mesangial cells also required intact Sp1 sites and was blocked by the GFA inhibitor DON. Thus, these results

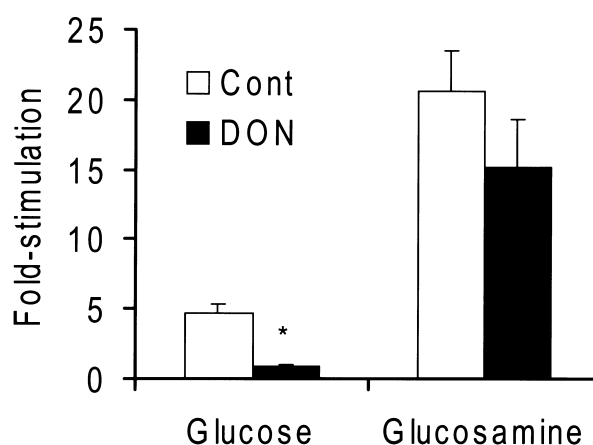


FIG. 8. Inhibition of GFA blocks the stimulation of PAI-1 promoter activity by high glucose. Mesangial cells were transiently transfected with a -740 to 44 PAI-1 promoter-luciferase reporter gene. The cells were treated for 4 days with 20 mmol/l or 1 mmol/l glucose or 2 mmol/l glucosamine or left untreated. During the final 48 h, 40 μ mol/l DON was added (■), and luciferase was assayed after 24 h in serum-free medium. The results are expressed as fold-stimulation by 20 mmol/l glucose or glucosamine compared with 1 mmol/l glucose or untreated cells, respectively. Results are means \pm SE ($n = 3$). * $P < 0.01$, 20 mmol/l glucose with DON vs. 20 mmol/l glucose alone.

support the concept that the hexosamine pathway participates in the induction of gene expression by high glucose in mesangial cells. Although luciferase activity may be augmented not only by increased transcription but also by effects on translation or protein stability, the minimal effect of glucosamine to stimulate luciferase activity using the Sp1 mutant PAI-1 promoter indicates that these latter mechanisms are unlikely to be significant. Because we have mutated only the Sp1 sites in the PAI-1 promoter, the current data do not exclude the possibility that other transcription factors act together with Sp1 to mediate the effects of glucosamine or high glucose. Sp1, a widely expressed zinc finger transcription factor, binds to GC- and GT-rich sites in most gene promoters (57). Although Sp1 is constitutively expressed (57), its activity is regulated by diverse factors. Indeed, the activity of Sp1 varies during development (58,59) and is modulated by growth factors (57), retinoblastoma protein (60), high glucose (61), cAMP (62), PKC (63,64), and extracellular signal-regulated kinase (ERK) (65).

Attempts to understand the effects of glucosamine have frequently focused on intracellular *O*-linked glycosylation (66). Treatment of cells with glucosamine or infusion of glucosamine in vivo increases cellular levels of UDP-GlcNAc, a substrate for both *N*- and *O*-linked protein glycosylation (19,25,43,44,66). However, glucosamine appears to block the *N*-glycosylation of at least some proteins (67,68). The relevance for gene expression of glucosamine-induced alterations in the *O*-glycosylation of extracellular proteins, such as proteoglycans, is unknown. In contrast, intracellular *O*-linked glycosylation has been postulated to influence protein-protein interactions, decrease protein phosphorylation, inhibit protein degradation, and facilitate cytoplasmic-nuclear protein transport (66). This type of *O*-glycosylation involves the addition of single *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) moieties to serine or threonine residues (66). The substrates are commonly multimeric nuclear proteins

such as Sp1, *c-myc*, serum response factor, RNA polymerase, and p62 nuclear pore protein (66) but also include cytosolic proteins such as cytoskeletal elements. In HeLa cells, Sp1 is modified by ~8 *O*-GlcNAc residues (45). Wheat germ agglutinin, which recognizes *O*-GlcNAc, interferes with transcriptional activation by Sp1 in vitro but not with DNA binding, suggesting that *O*-GlcNAc is concentrated in the NH₂-terminal transcriptional activation domain of Sp1 (45).

The augmentation of Sp1 DNA binding induced by glucosamine could have resulted from increased Sp1 protein stability secondary to *O*-glycosylation. A combination of glucose deprivation and high cAMP levels caused Sp1 to become hypoglycosylated and undergo proteasome-mediated degradation (46). This effect was reversed by glucose or glucosamine, which increased Sp1 *O*-glycosylation (46). However, there is no evidence yet that Sp1 protein stability is altered by glucosamine in the presence of physiological concentrations of glucose or by increasing glucose concentrations above normal. Other potential mechanisms of glucosamine-induced changes in Sp1 DNA binding, which are also relevant in the case of high glucose, include dephosphorylation of residues in the Sp1 DNA binding domain (61), release of Sp1 from an inhibitor (36), and increased Sp1 mRNA levels induced by PKC (64).

Sp3, a member of the Sp1 transcription factor family, may act as a negative regulator at Sp1 sites, although this effect is context dependent (47,48). For example, hypoxia has been reported to increase gene expression by decreasing Sp3 DNA binding and inhibitory activity (69). There was no evidence for this type of effect in the present study, since the amount of complex 2 (Fig. 5A), previously shown to represent Sp3 (47,48), was actually increased by glucosamine in the EMSAs.

It is unlikely that the 31% increase in Sp1 DNA binding fully explains the large increase observed in PAI-1 promoter activity induced by glucosamine. Thus, glucosamine may also have augmented transcriptional activation by Sp1 or affected interactions between Sp1 and other proteins. There are 4 transcriptional activation domains in Sp1 that interact with transcriptional cofactors, including subunits of the TF_{II}D and CRSP complexes (70–73). Recognized interactions between Sp1 and Smads (74), AP-1 (78), retinoblastoma protein (60,76), cyclin D (70), CBP/p300 (through progesterone receptors) (77), or other Sp1 molecules to form higher-order multimers (71) could all have been affected by glucosamine and merit further investigation.

At present we cannot distinguish whether the effects of glucosamine on the PAI-1 promoter are explained by direct *O*-glycosylation of Sp1, indirect effects on Sp1 mediated by *O*-glycosylation of other proteins, or perhaps other effects of glucosamine unrelated to *O*-glycosylation. In this regard, glucosamine-6-phosphate has recently been demonstrated to accumulate at high levels in the muscles of rats infused with glucosamine (75). These concentrations of glucosamine-6-phosphate exerted allosteric effects on hexokinase and glycogen synthase in vitro. Theoretically, allosteric interactions of glucosamine-6-phosphate with other proteins could also be responsible for the effects of glucosamine on Sp1.

Effects of glucosamine on Sp1 could have been mediated by changes in intracellular signaling. As noted above, Sp1 is activated by both conventional and atypical PKC isoforms (63,64) as well as ERK (65), a downstream target of PKC. Filippis et al. (79) suggested that glucosamine activates PKC in adipocytes. It has been reported that in mesangial cells,

12 mmol/l glucosamine—a higher concentration than used in the present experiments—caused membrane translocation of several PKC isoforms within hours (80). Whereas glucosamine required days rather than hours to activate the PAI-1 promoter, it will be important to discern whether PKC plays a role in mediating the effects of glucosamine on Sp1, since increased PKC activation is a major consequence of exposing cells to high glucose (7,12).

Analogous to the pivotal role played by TGF- β in many pathological conditions associated with scarring, in vivo and in vitro studies have implicated TGF- β 1 overexpression in the pathogenesis of diabetic nephropathy (8,15,81). Glucosamine increases TGF- β 1 mRNA and protein levels (25,26). However, our finding that a coexpressed dominant-negative type II TGF- β receptor failed to block activation of the PAI-1 promoter by glucosamine indicates that the effects of glucosamine on the PAI-1 promoter are not secondary to TGF- β secretion, but rather involve direct actions on transcription factors or intracellular signaling. TGF- β synthesized in response to glucosamine in the current experiments may not have become activated or may have been secreted in insufficient amounts to have an impact on PAI-1 promoter activity. On the other hand, our results do not rule out a contribution of hexosamine-induced TGF- β synthesis to the development of diabetic nephropathy in vivo.

In agreement with Chen et al. (36), we observed that activation of the PAI-1 promoter by high glucose depends, at least in part, on 2 Sp1 sites. However, Chen et al. found that up to 15 mmol/l glucosamine failed to increase PAI-1 mRNA levels after 48 h in vascular smooth muscle cells. In contrast, in glomerular mesangial cells, 2 mmol/l glucosamine increased both PAI-1 mRNA levels and PAI-1 promoter activity. There are several possible explanations for this discrepancy. First, the exposure to glucosamine in this study was for a longer duration (4 vs. 2 days), and we detected little activation of the PAI-1 promoter after a 2-day incubation with glucosamine. It is also possible that the higher concentrations of glucosamine used by Chen et al. (3.75–15 mmol/l), compared with 2 mmol/l in the present experiments, may have limited gene expression by depleting ATP or UTP. Finally, there may be cell-specific differences in the uptake or metabolism of glucosamine.

Upregulation of PAI-1 gene expression by glucosamine may be relevant to diabetic nephropathy. Renal biopsy specimens from patients with diabetic nephropathy show increased glomerular PAI-1 staining (6). Mesangial and endothelial cells maintained in high glucose in vitro secrete elevated amounts of PAI-1 and demonstrate increased PAI-1 mRNA levels (82–84). PAI-1 is the major physiological inhibitor of plasmin generation (85). High levels of PAI-1 are believed to favor the development of fibrosis in various types of inflammatory conditions (86) and glomerular diseases, including diabetic nephropathy (6,87), presumably because plasmin degrades ECM and activates other ECM-degrading metalloproteinases (85,86). In the current experiments, increases in UDP-HexNAc levels and PAI-1 gene expression were achieved by glucosamine. Elevations in hexosamine pathway flux in vivo mediated by high glucose concentrations could lead to important changes in the expression of PAI-1 and other genes. The ability of DON to block the effects of high glucose on the PAI-1 promoter further underscores the relevance of the hexosamine pathway in understanding the effects of high glucose.

In summary, we have shown that increasing flux through the hexosamine pathway with glucosamine enhances PAI-1 mRNA levels and PAI-1 promoter activity in mesangial cells. These changes in promoter activity are independent of TGF- β but are dependent on the integrity of two Sp1 sites in the PAI-1 promoter. Glucosamine increases Sp1 DNA binding by 31%, implying that glucosamine probably modifies additional aspects of Sp1 function. It is unclear whether glucosamine activates Sp1 directly by increasing its glycosylation or whether the effects on Sp1 are indirect. Activation of the PAI-1 promoter by high glucose also depends on these same Sp1 sites and is blocked by a GFA inhibitor, suggesting that the hexosamine pathway contributes to the effects of high glucose on the PAI-1 promoter in mesangial cells. Increased understanding of the hexosamine/Sp1 pathway could provide new targets for therapies directed at reducing the complications of diabetes.

ACKNOWLEDGMENTS

This work was supported by grants from the Juvenile Diabetes Foundation International and the Medical Research Council of Canada.

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